The Effect of adding Amino Acids to a Minimal Salts Medium on Slime Formation in Bacteria

An Honors Thesis (ID 499)

by

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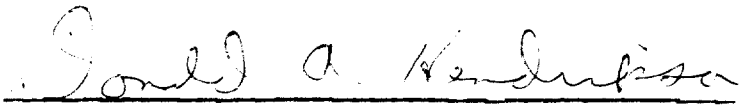

Ball State University

Muncie, Indiana

May, 1980

LITERATURE REVIEW

Various species of bacteria have been shown by numerous investigators to possess an extracellular coat or matrix which has, among its several functions, the mediation of attachment of the bacterial cell to solid surfaces or to other cells in the formation of microcolonies (1). This extracellular coat, composed of a sticky polysaccharide or glucan substance has been named the glycocalyx or "sweet husk" because of its chemical and physical properties (2,3).

This extracellular layer has been shown to stain heavily with both Ruthenium Red and Alcain Blue stains. This shows that the coat is composed, at least in part, of acidic polysaccharides (4,5). Electron microscopic observations describe this layer as mat-like, composed of many intertwining fibers. When observed in attached marine bacteria, the extracellular layer was found to have an average thickness of 15 nm. (6).

Electron microscopic studies of the polysaccharide layer of gram negative marine bacteria by Fletcher and Floodgate have shown this coat to be composed of two layers. The layer closest to the cell wall is an electron-dense primary acid polysaccharide. This layer can be further divided into an inner dense thin line and an outer fine-grained "fringe" region. The secondary acid polysaccharide layer, which is

found external to the primary layer is present only in attached bacteria. This layer is thought to arise from the primary layer after the initial attachment of the bacterial cell to the substratum has occured. The secondary acid polysaccharide layer is involved in the attachment of the primary layer to surrounding bacterial cells or to the substratum (6).

The precise chemical composition of the glycocalyx has been found, by Costerton, to vary with the composition of the individual fibers. The composition of these fibers is, in turn, determined by the most abundant carbohydrates available in the surrounding environment (7). A separate study concerning the chemical composition of the slime produced by a culture of Staphylococcus aureus found that the slime contained lactose, glycerol, and amino acids, as well as smaller amounts of glucose, uronic acid and galacturonic acid. The slime also contained protein, phosphorous, hexosamine, glucosamine anhydrorobitol, mono- and diphosphates and mannose (8).

The carbohydrate nature of the slime produced by Bacteriodes succinogenes and Ruminococcus flavefaciens in mixed culture was determined by the positive staining of the extracellular coats of these organisms with periodic acid thiosemicarbizide silver proteinate, a dye that shows the staining material contains periodate-reactive carbohydrate (9).

Trypsin and other proteolytic agents have been shown to

damage the glycocalyx on the cell surface. This evidence supports the hypothesis that the glycocalyx contains proteins as well as carbohydrates (10). The role of lectins, the proteins found in the glycocalyx, is described by Costerton (1) as being concerned with providing a link in the glycocalyx between adjacent bacterial cells. The lectins bind specifically to polysaccharides of a specific molecular structure. The lectins appear as highly ordered, globular protein subunits within the glycocalyx (11). The illustration in Figure I shows how the lectins and polysaccharides of the glycocalyx are physically ordered so that they act to connect adjacent bacterial cells.

The glycocalyx of the bacterial cell is produced by the cell itself in an energy-requiring process. The cell expends the energy out of necessity since the glycocalyx affords it several survival advantages in its natural environment (1,3). The synthesis of low molecular weight polysaccharides which are intermediates of the larger polysaccharides composing the fibers of the glycocalyx has been shown to occur in a membtane fraction. Northcote showed that these polysaccharides were attached to proteins which acted as transfer agents during the transfer of the molecule across the cell membrane. During the movement across the cell membtane the glycoproteins are believed to be attacked by specific proteases or transglycosidases contained

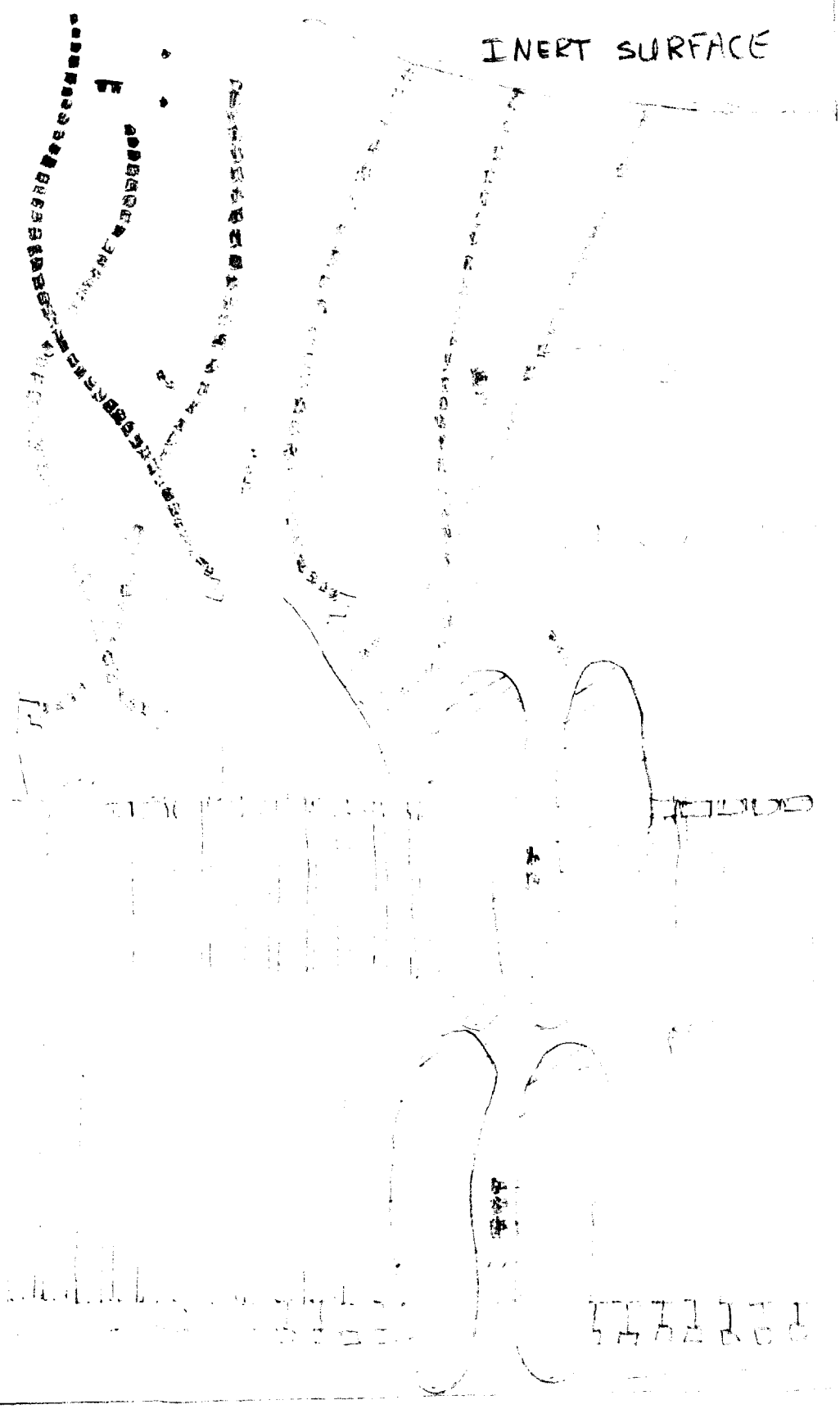

Figure I
Attachment of a Bacterium to an Inert Surface
Redrawn from Costerton, et.al., Scientific American, 238 (1), pp. 86-95.

in the area between the cell wall and the plasmalemma. The synthesis of the polymers composing the glycocalyx occurs within the cell membrane. After later detachment of the protein carrier molecule the slime polymer is secreted to the extracellular area (12).

The production of slime layers by bacteria has been shown to require the presence of sucrose in the medium (13). Streptococcus mutans, the organism responsible for the production of dental caries posesses three enzymes which function in the breakdown of sucrose. These enzynes; invertase, glucosyltransferase, and fructosyltransferase, have been localized near the plasma membrane or enmeshed within the fibers of the glycocalyx (1,14, 15). Invertase splits the sucrose molecule into a molecule of glucose and one of fructose. Glucosyltransferase, after splitting the sucrose molecule in the same way, releases the fructose and polymerizes the glucose to form a large polymer known as a glucan. Fructosyltransferase also splits the disaccharide into glucose and fructose but releases the glucose and uses the fructose in the formation of a polymer of smaller size and molecular weight than the glucans (1).

The location of these enzymes is related to the role they play in the growth and expansion of the glycocalyx (14). Their activities make available the building block monosaccharides

which are the basic components of the polysaccharide fibers.

The formation of the glycocalyx by the bacterial cell is, as previously stated, an energy requiring process. The cell continues to expend the energy necessary for the formation of the glycocalyx because the polysaccharide fibers provide a means of protection for the cell as well as having several other advantageous functions. Costerton (1) states, "The glycocalyx is essential to the biological success of most bacteria in most of the varied natural environments in which they are observed".

The glycocalyx functions to protect the bacterial cell against predatory bacteria, protozoans and viruses, as well as harmful ions and molecules, host antibodies, antibiotics and physical stresses (1,11). The glycocalyx accomplishes this by decreasing the already limited penetrability of the cell wall and by acting as an ion exchange resin by virtue of its charged carbohydrate and protein groups (11). In its action as an ion exchange resin, the glycocalyx can both prevent the entry of harmful molecules and ions and can trap beneficial nutrient molecules (16).

By attaching bacterial cells to one another the glycocalyx groups them into units which can more effectively carry out physiological processes (1). The grouping of cells by the slime layer also results in the establishment of microniches or microcolonies, allowing bacteria to avoid danger and stress

in environments that they would otherwise tolerate poorly (16). The examination of attached bacterial populations in a free flowing alpine stream by Geesey, et. al. showed that the glycocalyx surrounding the bacteria served several purposes. Among these were: providing protection from the physical force of the flowing water, providing a means of attachment and access to a source of food such as an algal cell or another primary producer and providing protection from other predatory bacteria or protozoans which may be present in the stream (16).

In other situations, the glycocalyx, by allowing attachment of the bacteria to various surfaces may promote infection and bacterial disease or have other detrimental effects such as the production of dental caries by Streptococcus mutans (17, 18, 19, 20).

The adhesion of the bacterial cell to various surfaces as well as to other cells is obviously one of the major functions of the glycocalyx. The process of adhesion is described as occuring in two steps by Marshall, Stout, and Mitchell (17). In their study using gram negative marine bacteria, two stages of sorption or adhesion were postulated to occur. In the first or reversable stage the bacteria are held weakly near the surfaces to which they are attaching. During this stage the bacteria still exhibit Brownian

motion and can be removed easily from the surface by washing with 2.5% NaCl. The forces holding the bacteria near the surface during this stage are explained by Marshall et.al. as being a combination of the London-Van der Waals attractive energies between the surfaces and the electrical repulsive energies resulting from the overlapping ionic atmospheres around the surfaces (17).

The second stage of sorption, the irreversable stage, occurs, according to Marshall et. al., when polymeric bridging between the bacterial surface and the substrate forms. This bridging serves to overcome the repulsive forces described for stage one (17).

A second mechanism postulated for adhesion concerns the interaction of the carbohydrates in the glycocalyx with other carbohydrates on the surface of an adjacent cell. In this mechanism, adhesion occurs when hydrogen bonds form between glucose units on the two surfaces. A weakness of this model is the necessity for the formation of a very large number of hydrogen bonds to produce effective adhesion (21)

A simpler and more widely applicable proposal states that adhesion occurs as a result of the interaction of enzymes and substrates on adjacent surfaces (22). The interaction of antigen and antibody-like substances on adjacent cell surfaces has also been proposed as a mechanism (23).

The adhesion of bacteria through slime layers in nature occurs in many locations and situations. Adhesion to cellulose fibers by Ruminococcus albus aids that bacteria in the digestion of the cellulose, thereby making the nutrients in plants more readily available to it (24). The adhesion of marine bacteria to surfaces is stimulated by the low levels of carbon in seawater. By adhering to surfaces, the bacteria are exposed to more carbon sources and thus can more easily supply their metabolic needs (17). The adhesion of bacteria such as Streptococcus bovis to epithelial cell surfaces in the rumen of cattle can increase their access to food supplies and can also protect them from harmful molecules, ions, or antibodies present in the secretions passing by them (5,18). The adhesion of these bacteria in this location may also increase their pathogenicity resulting in various diseases such as the frothy feedlot bloat produced by Streptococcus bovis (5).

The adhesion of bacteria to various tissue surfaces in humans through slime formation also results in many bacterial infections. Bacteriodes fragilis is the major organism found in intra-abdominal sepsis. The attachment of this organism to the abdominal endothelium is mediated by a glycocalyx (20). The pathogenicity of Klebsiella pneumoniae is also increased by the presence of a glycocalyx by which it is able to attach and result in pneumonia (25).

The attachment of bacteria to surfaces through slime formation has been noted in several other diseases and infections such as the endocarditis produced by the adherence of Streptococcus sanguis to traumatized heart valves (26,27, 28).

The majority of the research reviewed in this area was concerned with the role of the glycocalyx of Streptococcus mutans in initiating and maintaining the adherence of the bacterium to tooth enamel. The adhesion of these organisms to the tooth is represented in the diagrams in Figures II and III. The initial adhesion of the S. mutans cell to the enamel surface occurs as dietary sucrose is broken down to form glucose and fructose which are formed into polymers by the glucosyltransferases and fructosyltransferases of the bacterium. After attachment occurs, large amounts of lactic acid are produced by enzymes within the glycocalyx. This results in demineralization and eventual destruction of the enamel (14,18,24,29).

Control of the various situations resulting from the adhesion of bacteria to various surfaces may be approached in several ways. The production of glycocalyx has been shown to be dependent upon the optimum temperature and pH of the particular organism producing the slime. Therfore, the optimum pH and temperature for a particular organism could be varied according to the amount of slime desired by the experimenters. The adhesion of an organism to a substrate could, theoretically, be prevented if the values of these

Figure II
Use of Sucrose by Streptococcus mutans
Redrawn from Costerton et.al., Scientific American, 238(1), pp. 86-95.

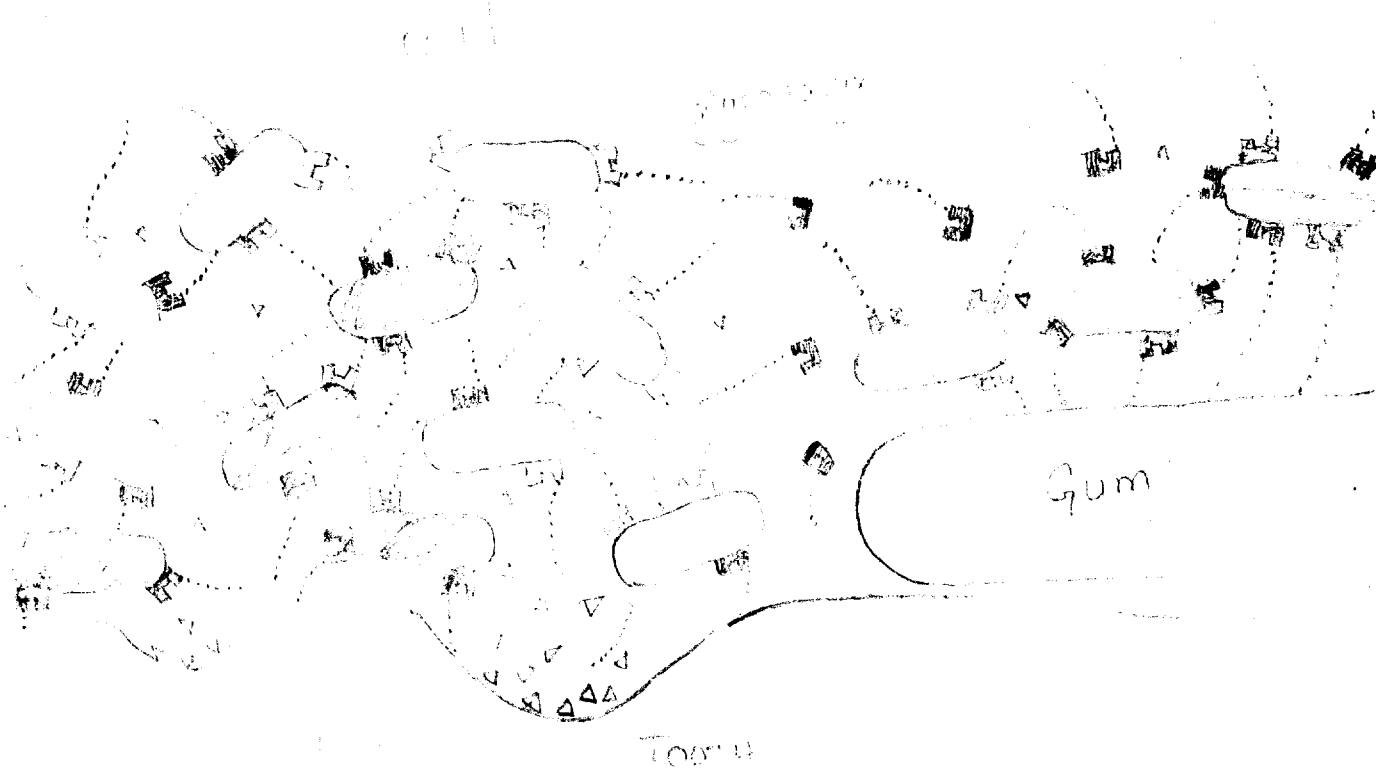

Figure III
Formation of Dental Plaque

Redrawn from Costerton, et.al., Scientific American 238(1), pp. 86-95.

variables were known for the organism involved. The difficulty in employing this approach in many instances is both the inability to change the normal temperature and pH in the location necessary and the inability to maintain either variable at the new value for any length of time.

Since the adhesion through lectins of the glycocalyx of a given organism to its specific substrate is controlled by the molecular characteristics of the substrate, the lectins, and the glycocalyx, the prevention of adhesion is a logical approach to take in preventing infection by a particular bacterium (1). Costerton outlines three ways that a new class of antibiotics could be used to interfere with glycocalyx formation or function for specific bacteria. The synthesis of the glycocalyx could be interfered with by inhibiting the polymerase that links the sugar subunits together to form the polysaccharide chains. This could be accomplished by exposing the polymerases of the slime layer to a compound that competes with the sugar substrate for the enzyme's active site. A second point for interference is at the active site of the lectins which attach the bacterial cell to the substrate or to the host cell. By exposing the bacterium to compounds which compete for the site on the lectins through which attachment to other lectins or to the substrate occurs, the adhesion of the bacterium to its specific site of attachment could be prevented. The final alternative discussed by Cos-

terton is the blockage of the receptor sites for the polysaccharide fibers on the host cells. By using this approach, the antibiotic used would not have to enter the host cell or the bacterial cell. This would avoid both toxicity to the host and the development of bacterial resistance to the antibiotic treatment. Costerton states that, initially, bits of the slime, subunits of the strands, or analogs of the sugars would be effective in preventing adhesion when used with this goal in mind (1).

The glucosyltransferases of Streptococcus mutans have been shown to produce both α1-3 and α1-6 linked glucose strands. The adhesion and slime production are increased when both of these linkages are included in the glucan structure (14, 30). An effective anticaries vaccine should, therefore, effectively inhibit both the glucosyltransferases forming the α1-3 links and those forming the α1-6 links since it is the combination of these enzyme activities that results in the greatest slime production and therefore the greatest virulence for the bacteria (14).

The production of slime by bacterial organisms in industrial cooling systems is an important area of study since the production of slime in these locations can lead to many problems such as lowered operating efficiency. Control of the production of slime in these locations is usually done by chemical treatment. Various chemicals have been used in

this effort such as chlorine-ammonia, chlorine dioxide, hypochlorite and calcium (8).

The treatment with calcium is reported to have a hardening effect on the slime. An excess of this divalent ion ties up the negatively charged ends of the polysaccharide so that the glycocalyx adheres to itself rather than to other surfaces (8). Chlorine dioxide is often used instead of chlorine since its oxidizing power is 2.6 times as great as that of chlorine and since it can penetrate the glycocalyx to reach the bacteria much better than can the chlorine molecule. The use of chlorine also entails another problem because its high reactivity often causes it to become involved in side reactions which reduce its effectiveness (4). The chlorine dioxide, since it is able to reach the bacterial cell, kills the cell and so removes the primary cause of the slime problem (4).

Hypochlorite acts on the slime fibers by cleaving the bond at the C_2-C_3 position of the D-glucose units by oxidation. This causes a random depolymerization of the glycocalyx and results in production of a variety of high molecular weight products (8). Since destruction of the slime, rather than inhibition of the growth of the bacteria is the desired result of the chemical treatment, the hypochlorite is the most effective chemical in achieving this aim (8).

The formation of the slime layer is also affected by many other chemicals. The formation of the glycocalyx increases when the sulfur source in the medium is changed from sulfate to sulfite (22), when the carbohydrate or its concentration is changed (7), or when the temperature of the culture is changed (31, 32, 33). High concentrations of sucrose and low concentrations of glucose have also been found to increase slime production (7,17). The presence of calcium and magnesium ions has also been found to be essential for slime production since, in the absence of these ions, part of the cell surface necessary for adhesion was removed, resulting in the inability of the cells to either adhere or aggregate (21, 17). It is hypothesized that these ions are incorporated as a stabilizing factor into the mat of fibers in the glycocalyx. The simple addition of these ions to a medium on which Pseudomonas strain R3 was being grown did not stimulate its adhesion (17).

The understanding of the role each of these stimulatory factors plays in the production of the glycocalyx combined with the development of various types of antibiotic therapy against the production of the slime will contribute substantially to both our understanding of the nature and function of the slime layer and to our ability to control its detrimental effects.

MATERIALS AND METHODS

The research involved in this study was carried out in the lab of Dr. Donald A. Hendrickson, Cooper Life Sciences Building, room 34.

In the first section of this study three different cultures of slime-forming, gram negative bacteria were established by isolating the cultures from water samples taken from the White River in Muncie, Indiana. The cultures were initially isolated on TGEA and MacConkey's agar. Slime formation was established if tenacity was exhibited when the culture was touched with an innoculating loop.

The cultures were then identified using a series of innoculations onto differential bacteriological testing media. The tests used and the results obtained which allowed the identification of the three isolates are listed in Table I in the appendix.

Following isolation and identification of the cultures, the bacteria were plated onto a minimal salts medium using sulfate as the sulfure source. This medium had been used previously (34) in an attempt to cause slime forming bacteria to form the most abundant slime layer. An increase in slime formation has been reported when the sulfur source is changed from sulfate to sulfite (22). A sulfate source

was used in this study since the sulfates necessary for the medium were more available than were the sulfites.

The procedure for preparing the basic minimal salts-carbohydrate medium is outlined in Figure IV. The instructions for the preparation of the minimal salts medium using sulfite as the sulfur source are also included and may be found in Figure V. The calculations involved in determining the amounts of the various salts included in the media are not given in this paper but are reported by Taylor (34).

Three different carbohydrates, each in two concentrations were substituted for each other in this medium. The carbohydrates and the concentrations used are the following: 0.5% glucose (5.0 g/liter), 1.0% glucose (10.0 g/liter), 2.0% sucrose (20.0 g/liter), 5.0% sucrose (50.0g/liter), 2.0% lactose (20.0 g/liter), and 5.0% lactose (50.0 g/liter). The amount of growth and degree of slime formation which occured with the use of each of these sugar concentrations were compared.

One of the objectives of this study was the determination of the possible role of amino acids in increasing the amount of slime produced by a culture. To study this a mixture of pure D and L amino acids was substituted for the normal nitrogen source, $(NH_4)_2SO_4$, in the minimal salts medium. The moles of nitrogen added in the amino acid mixture was equal to the number of moles usually added as $(NH_4)_2SO_4$ in one set of trials and was of lower molarity in a second

Figure IV

Minimal Salts-Carbohydrate Medium with Sulfate Preparation Procedure

Carbohydrates

Use one of the following per liter of medium.

0.5% glucose	5.0 grams
1.0% glucose	10.0 grams
2.0% sucrose	20.0 grams
5.0% sucrose	50.0 grams
2.0% lactose	20.0 grams
5.0% lactose	50.0 grams
Agar	15.0 grams
0.5 M Na_2HPO_4	5.0 ml.
Concentrated minimal salts	20.0 ml.

Dissolve the carbohydrate and agar in 750 ml. of deionized water. In 250 ml. water dissolve the base and the $(NH_4)_2SO_4$. Sterilize the medium in two flasks. One should contain the carbohydrate, agar and phosphate. The other should contain the base and ammonium sulfate.

All of the carbohydrate media were made at the same time so the base-ammonium sulfate fractions were combined and a graduated cylinder which had been pre-sterilized was used to pour the right amount of minimal salts medium into the flask containing the carbohydrate and agar. When the medium had cooled the fractions were combined and poured into petri plates.

FIGURE IV (continued)

To 1000 ml. deionized water add:

1.0 gram $(NH_4)_2SO_4$

5.0 ml. of 0.5M Na_2HPO_4

20.0 ml. Concentrated base stock solution

The concentrated base stock solution is made as follows:

To 100 ml. deionized water add:

Nitrilotriacetic acid	1.0000g.
$MgSO_4$	1.4450g.
$CaCl_2$ 2 H_2O	0.3335g.
$(NH_4)_6Mo_7O_{24}$ $4H_2O$	0.0009g.
$FeSO_4$ $7H_2O$	0.0099g.
Metals "44"	5.0 ml.

To prepare this solution dissolve the nitrilotriacetic acid in the water and neutralize it with 0.73 grams KOH. The rest of the ingredients are then added.

Metals "44" Solution Preparation

To 30 ml. of deionized water add:

EDTA	0.0750g.
$ZnSO_4$ $7H_2O$	0.3285g.
$FeSo_4$ $7H_2O$	0.1500g.
$MnSO_4$ H_2O	0.0462g.
$CuSO_4$ $5H_2O$	0.0118g.
$Co(NO_3)_2$ $6H_2O$	0.0074g.
$Na_2B_4O_7$ 10 H_2O	0.0053g.

Add a few drops of H_2SO_4 to prevent precipitation.

Figure V

Minimal Salts-Carbohydrate Medium --Sulfite Preparation

In 1000 ml. :

One of the following sugars:

0.5% glucose	5.0 grams
1.0 % glucose	10.0 grams
2.0% sucrose	20.0 grams
5.0% sucrose	50.0 grams
agar	15.0 grams
1.0 M Na-pyrophosphate -HCl buffer	5.0 ml.

(50 ml. of 44.58 g pyrophosphate in 100 ml)
plus
(52.35 ml. of 2M HCl)
dilute this to 200 ml.

concentrated minimal salts base	20.0 ml.
$(NH_4)_2SO_3H_2O$	1.02 grams

This medium is made using the same procedure used for the sulfate medium, as described in Figure IV.

FIGURE V (continued)

In 1000 ml. deionized water:

add 1.02 grams $(NH_4)_2SO_3H_2O$

5.0 ml. sodium pyrophosphate-HCl buffer

20.0 ml. concentrated base (stock solution)

Concentrated base stock solution is made as follows:

To 100 ml. deionized water add:

nitrilotriacetic acid	1.0000g.
$MgSO_3$ $6H_2O$	2.5000g.
$CaCl_2$ $2H_2O$	0,3335g.
$(NH_4)_6Mo_7O_{24}$	0.0009g.
$FeCl_2$ $4H_2O$	0.0707g.
Metals "44" solution	5.0 ml.

To prepare this solution, dissolve the nitrilotriacetic acid in the water and neutralize it with 0.73 grams KOH. The rest of the ingredients are then added.

Metals "44" Solution preparation

To 30 ml. of deionized water add:

EDTA	0.0750g.
$ZnSO_3$ $2H_2O$	0.2205g.
$FeCl_2$ $4H_2O$	0.1071g.
$MnSO_3$	0.0369g.
$CuCl_2$	0.0064g.
$Co(NO_3)_2$ $6H_2O$	0.0074g.
$Na_2B_4O_7$ $10H_2O$	0.0053g.

Add a few drops of H_2SO_4 to prevent precipitation.

set of trials. The list of amino acids and the molar amounts added of each are listed in Table II of the appendix. In the series of trials using the amino acid mixture with nitrogen molarity equal to that of the $(NH_4)_2SO_4$, each amino acid was weighed from a pure dry sample and added to 6 ml. of potassium phosphate buffer, pH 7.2. The weights of the amino acids added to this mixture are also listed in Table II.

The innoculation of the minimal salts medium was done according to the following procedure. Each of the isolates was transferred to a tube containing sterile buffered water and mixed. A drop of the mixture was placed on a Petroff-Housser counting chamber. The number of cells per grid was counted. These counts were repeated ten times for each isolate and averaged. The average number of cells per grid was multiplied by $2x10^7$ to give the number of cells per milliliter. Using these counts, the original mixture of each isolate was dilluted by serial dilution so that the final tube contained approximately 10^2 to 10^3 cells per milliliter. These dilutions were then used for all the innoculations.

Using the final dilution, 0.1 ml. of the mixture was delivered by sterile pipettes to the agar surface in the petri plates. A curved glass rod was then sterilized and used to spread the drop across the agar. The plates were

* Note: The molarity reported for the amino acid mixtures throughout this paper is the total Nitrogen molarity contained in the mixtures. For the molarity of the amino acids please see Table II in the appendix.

then inverted and incubated at 37°C for seven days.

In each of three trials the three isolates were plated onto each of six different media, each containing a different sugar or sugar concentration in which $(NH_4)_2SO_4$ was the nitrogen source. In the same three trials the three organisms were plated onto each of the six minimal salts medium plates in which the amino acid mixture (N= 1.4×10^{-6} M) had been substituted for the $(NH_4)_2SO_4$ nitrogen source.

The same procedure was repeated using the amino acid mixture in which the total nitrogen molarity was equal to the nitrogen molarity using $(NH_4)_2SO_4$ (7.57×10^{-3} M).

After seven days the following characteristics were noted for each culture plate: average colony size, measured with a metric ruler, colony color and nature, raised or depressed center, number of colonies per square centimeter, presence of a capsule using a negative stain, and tenacity, which was determined by touching the colony with an innoculating needle. In order to enable the comparison of the amount of slime production exhibited by the various cultures the tenacity present on each plate was immediately assigned a number on a "tenacity scale" ranging from 1 (little or no tenacity) to 10 (very strong tenacity). This system was used so that the comparison of slime production between culture plates could be

made on as objective a basis as possible. The data collected for the two sets of trials on the above characteristics is summarized in Table III and Table IV of the appendix.

RESULTS

All three isolates grew on the minimal salts-carbohydrate medium using either the $(NH_4)_2SO_4$ or the amino acid mixtures as the nitrogen source, though none of the isolates produced as much slime on the minimal salts medium as they did when initially isolated on TGEA and MacConkey's agar.

Isolate F exhibited the greatest amount of growth when grown on medium containing 1% glucose. The tenacity shown by isolate F was greatest when 1% glucose or 5% sucrose was contained in the medium. Tenacity was improved for isolate F when the 1.4×10^{-6} M amino acid mixture was substituted for the $(NH_4)_2SO_4$ when the medium contained either 1.0% glucose or 5.0% sucrose.

Isolate A exhibited the best growth with 5.0% sucrose in the medium. The tenacity exhibited by isolate A was greatest using 5.0% sucrose at both concentrations of amino acids. Much slime was also produced when isolate A was grown on medium with 2.0% sucrose or 1.0% glucose at both amino acid concentrations. The substitution of the amino acid mixture for the $(NH_4)_2SO_4$ increased the tenacity exhibited by isolate A when the following sugar and amino acid concentrations were used: 0.5% glucose (1.4×10^{-6} M amino acids),

2.0% sucrose (1.4×10^{-6} M and 7.5×10^{-3} M amino acids), and 5.0% sucrose (1.4×10^{-6} M and 7.5×10^{-3} M amino acids).

Isolate H exhibited the highest degree of growth when grown on medium containing either 1.0% glucose or 2.0% lactose. The greatest slime productin was exhibited by isolate H when grown on medium with 2.0% lactose and 7.5×10^{-3} M amino acids. The tenacity exhibited on medium with 5.0% sucrose and 1.4×10^{-6} M amino acids was only slightly less than this. The substitution of the amino acids mixture for the $(NH_4)_2SO_4$ produced an increase in slime production for isolate H only for the following concentrations of carbohydrate and amino acids: 0.5% glucose (7.5×10^{-3} M and 1.4×10^{-6} M amino acids), 1.0% glucose (7.5×10^{-3} M amino acids) and 2.0% sucrose (1.4×10^{-6} M amino acids).

Growth for all three isolates was better when the amino acid concentration was higher. The degree of tenacity exhibited by each of the three isolates did not change consistently either with a higher amino acid concentration or with the substitution of the amino acid mixture at either concentration of nitrogen for the $(NH_4)_2SO_4$ as the nitrogen source.

CONCLUSIONS

The substitution of the amino acid mixture for the $(NH_4)_2SO_4$ did not result in a clear increase in tenacity for all or even the majority of the carbohydrate concentrations used for any one of the three isolates. Therefore, the data collected in this study did not support the suggestion of Roseman (13) that the nutrient missing in the minimal salts-carbohydrate medium which was necessary to enhance the slime production to the greatest extent was an amino acid.

It is possible that the mixture of amino acids used in this study did not include an amino acid necessary for the greatest production of slime. It is also possible that this mixture did not include a D form of one of the amino acids and that this particular form of the amino acid was a necessary component of or precursor of the slime formed. Please see Table II of the appendix for this information.

The decreased growth and the overall decrease in slime production observed for all three isolates on the minimal salts-carbohydrate medium as compared to the growth and slime production exhibited on MacConkey's agar and TGEA may be attributed to the decreased availability of many of the nutrients present in the TGEA and MacConkey's agar other than amino acids, which were not present in the minimal

salts medium.

The generally lower growth present for all three isolates on those plates in which one of the amino acid mixtures had been substituted for the $(NH_4)_2SO_4$ may have resulted from the unavailability of the nitrogen present in the more simple form as $(NH_4)_2SO_4$. The only nitrogen present in the medium where the amino acid mixture was used was in a more complex form as part of the amino acids, thus making it somewhat less available for easy access and use in the bacterial cell's other nitrogen-requiring metabolic activities.

From the data presented as results of this study, it can be seen that the substitution of amino acids in either of two concentrations, 7.5×10^{-3} M or 1.4×10^{-6} M, did not result in an increase in slime production for any one of the isolates when grown on all six different sugar concentrations in the minimal salts medium. The hypothesis of this study that substituting the nitrogen source in the medium with a mixture of purified amino acids would result in greater slime production was supported by each of the isolates when grown on some of the media used but was not supported with each isolate's growth on every medium. Isolate F's growth supported the hypothesis when grown on 1.0% glucose and 5.0% sucrose using both concentrations of amino acids. The slime production of isolate A increased when grown on medium containing 2.0% sucrose or 5.0% sucrose at both concentrations of amino acids and on medium containing 0.5% glucose with 1.4×10^{-6} M amino acids. Isolate H's growth and slime production supported the hypothesis when it was grown on medium containing 0.5% glucose

with 1.4×10^{-6} M amino acids, 1.0% glucose with 7.5×10^{-3} M amino acids and with 0.5% glucose at both concentrations of amino acids.

APPENDIX

Table I

Test	Isolate A	Isolate F	Isolate H
gram stain	-	-	-
lactose	-	+	+
catalase	-	-	+
oxidase	+	+	-
indole	-	-	+
MR	-	+	+
VP	+	-	-
H_2S	-	+	-
motility	+	+?	-
nitrate reduc.	+	+	-
urease	-	-	-
rhamnose	+	-	+
raffinose	+	+	-
fluorescence	-	-	+
pigment	brown	yellow	green, diffusable
citrate	+	-	+
inositol	+	+	-
mannitol	+	+	+
tenacity	+	+	+

Based on the above test results the following identifications of these isolates are proposed to be: for Isolate A, Enterobacter aerogenes, for Isolate F, Enterobacter hafniae, and for Isolate H, Pseudomonas species.

Table II

Amino Acid	Moles/ l. in Soln. I	Moles/ l. in Soln II	grams in Soln. II
D-alanine	1.04×10^{-8}	0.00	0.00
L-alanine	1.04×10^{-8}	6.25×10^{-4}	0.01172
L-arginine	1.04×10^{-8}	3.125×10^{-4}	0.01146
D-aspartate	1.04×10^{-8}	0.00	0.00
L-aspartate	1.04×10^{-8}	6.25×10^{-4}	0.01752
Cysteine	1.04×10^{-8}	3.125×10^{-4}	0.00796
Glutamate	1.04×10^{-8}	3.125×10^{-4}	0.00968
L-histidine	1.04×10^{-8}	3.125×10^{-4}	0.0102
L-isoleucine	1.04×10^{-8}	3.125×10^{-4}	0.00862
L-leucine	1.04×10^{-8}	3.125×10^{-4}	0.00862
D-lysine	1.04×10^{-8}	0.00	0.0
L-lysine	1.04×10^{-8}	6.25×10^{-4}	0.0192
D-methionine	1.04×10^{-8}	0.00	0.00
L-methionine	1.04×10^{-8}	6.25×10^{-4}	0.0196
D-phenylalanine	1.04×10^{-8}	0.00	0.00
L-phenylalanine	1.04×10^{-8}	6.25×10^{-4}	0.0216
L-proline	1.04×10^{-8}	3.125×10^{-4}	0.0076
D-serine	1.04×10^{-8}	0.00	0.00
L-serine	1.04×10^{-8}	6.25×10^{-4}	0.01384
L-threonine	1.04×10^{-8}	0.00	0.00
D-tyrosine	1.04×10^{-8}	0.00	0.0
L-tyrosine	1.04×10^{-8}	6.25×10^{-4}	0.02384
L-valine	1.04×10^{-8}	3.125×10^{-4}	0.0077
glycine	1.04×10^{-8}	3.125×10^{-4}	0.00494

Table III

Characteristic	Organisms A	F	H
Cell Morphology	bacilli, single	bacilli, some diplo	bacilli, single
Colony color	yellow-white	dark yellow	dk. brown-green
Colony nature	opaque	opaque	opaque
Center	raised	raised	raised
Capsule	no	no	no

The above data are results of the trials using the 1.4×10^{-6} M amino acid mixture. The results on these characteristics are not reported separately for each different sugar used in the medium since these characteristics remained constant throughout the trials.

Characteristic	Isolate A	F	H
Colony color	light yellow	dark yellow	brown-green
Colony nature	opaque	opaque	opaque
Center	raised	raised	raised
Capsule	raised	raised	raised

The above characteristics are the result of the trials performed using the amino acid mixture with nitrogen molarity of 7.5×10^{-3} M. As explained above, these characteristics are reported as a group for these trials since they did not change as the sugar used in the medium was changed.

Table IV

Part I. Results for Isolate A

Medium		Medium	
0.5%glucose	colonies pinpt.-2.0mm. 31/sq. cm. tenacity 3	0.5% glucose ($1.4x10^{-6}$M amino acids)	colonies pinpt.-2.0 mm. 18/sq. cm. tenacity 5
1.0% glucose	colonies pinpt.-4.0 mm, 13/sq. cm. tenacity 6	1.0%glucose ($1.4x10^{-6}$M amino acids)	colonies 0.25-0.30 mm. 16/sq. cm. tenacity 4
2.0% sucrose	colonies pinpt. -3.0 mm. 13/sq. cm. tenacity 4	2.0% sucrose ($1.4x10^{-6}$M amino acids)	colonies pinpt. -4.0 mm. 20/sq. cm. tenacity 6
5.0% sucrose	colonies pinpt. -3.0 mm. 19/sq. cm. tenacity 8	5.0% sucrose ($1.4x10^{-6}$M amino acids)	colonies pinpt. -2.0 mm. 20/sq. cm. tenacity 7
2.0% lactose	colonies pinpt. to 6.0 mm. 12/sq. cm. tenacity 2	2.0% lactose ($1.4x10^{-6}$M amino acids)	colonies pinpt. -3.0mm. 18/sq. cm. tenacity 1
5.0% lactose	colonies pinpt. 50/sq. cm. tenacity 2	5.0% lactose ($1.4x10^{-6}$M amino acids)	colonies pinpt. -2.5mm. 20/sq. cm. tenacity 1.5

Results of the second set of Trials

Medium			
0.5%glucose	sheet of growth tenacity 2	0.5% glucose ($7.5x10^{-3}$M amino acids)	sheet of growth tenacity 1
1.0% glucose	sheet of growth tenacity 8	1.0% glucose ($7.5x10^{-3}$M amino acids)	sheet of growth tenacity 1
2.0% sucrose	sheet of growth tenacity 6	2.0% sucrose ($7.5x10^{-3}$M amino acids)	sheet of growth tenacity 8
5.0%sucrose	sheet of growth tenacity 7	5.0% sucrose ($7.5x10^{-3}$ M amino acids)	sheet of growth tenacity 8
2.0%lactose	no growth	2.0% lactose ($7.5x10^{-3}$M) amino acids)	sheet of growth tenacity 5
5.0% lactose	no growth	5.0%lactose ($7.5x10^{-3}$M amino acids)	sheet of growth tenacity 5

Table IV

Part II. Results for Isolate F

Medium		Medium	
0.5% glucose	colonies 0.5-1.0 mm. 38/sq. cm. tenacity 3	0.5% glucose (1.4×10^{-6} M amino acids)	colonies pinpt. -4.0 mm. 11/sq. cm. tenacity 4
1.0% glucose	colonies 0.1-5.0 mm. 28/sq. cm. tenacity 4	1.0% glucose (1.4×10^{-6} M amino acids)	colonies 0.1-1.5 mm. 4/sq. cm. tenacity 7
2.0% sucrose	colonies pinpt. -3.0 mm. 13/sq. cm. tenacity 4	2.0% sucrose (1.4×10^{-6} M amino acids)	colonies pinpt.-2.5 mm. 15/sq. cm. tenacity 3
5.0% sucrose	colonies pinpt. -2.0 mm. 9/sq. cm. tenacity 2	5.0% sucrose (1.4×10^{-6} M amino acids)	colonies pinpt.-1.5 mm. 11/sq. cm. tenacity 6
2.0% lactose	colonies 0.5-2.0 mm. 10/sq. cm. tenacity 5	2.0% lactose (1.4×10^{-6} M amino acids)	colonies 1.0-4.0 mm. 18/sq. cm. tenacity 6
5.0% lactose	colonies 0.25-2.0 mm. 30/sq. cm. tenacity 5	5.0% lactose (1.4×10^{-6} M amino acids)	colonies 0.5-4.0 mm. 5/sq. cm. tenacity 5

Results for Second Set of Trials

Medium		Medium	
0.5% glucose	sheet of growth tenacity 3	0.5% glucose (7.5×10^{-3} M amino acids)	sheet of growth tenacity 2
1.0% glucose	sheet of growth tenacity 8	1.0% glucose (7.5×10^{-3} M amino acids)	sheet of growth tenacity 8
2.0% sucrose	sheet of growth tenacity 8	2.0% sucrose (7.5×10^{-3} M amino acids)	sheet of growth tenacity 7
5.0% sucrose	sheet of growth tenacity 8	5.0% sucrose (7.5×10^{-3} M amino acids)	sheet of growth tenacity 5
2.0% lactose	sheet of growth tenacity 4	2.0% lactose (7.5×10^{-3} M amino acids)	sheet of growth tenacity 5
5.0% lactose	sheet of growth tenacity 4	5.0% lactose (7.5×10^{-3} M amino acids)	sheet of growth tenacity 4

Table IV
Part III. Results for Isolate H

Medium		Medium	
0.5% glucose	colonies 0.75-1.0mm. 21/sq. cm. tenacity 4	0.5% glucose (1.4×10^{-6} M amino acids)	colonies 4-7 mm. 1 large/sq. cm. tenacity 7
1.0% glucose	colonies pnpt.-4.5 mm. 34/sq. cm. tenacity 7	1.0% glucose (1.4×10^{-6} M amino acids)	colonies 0.25-2.5 mm. 25/sq. cm. tenacity 3
2.0% sucrose	colonies pinpt.-2.0mm. 6/sq. cm. tenacity 5	2.0% sucrose (1.4×10^{-6} M amino acids)	colonies pinpt.-3.0mm. 6/sq. cm. tenacity 6
5.0% sucrose	colonies pinpt. 25/sq. cm. tenacity 7	5.0% sucrose (1.4×10^{-6} M amino acids)	colonies pinpt. 20/sq. cm. tenacity 5
2.0% lactose	colonies 0.25-0.5mm. 58/sq. cm. tenacity 3	2.0% lactose (1.4×10^{-6} M amino acids)	colonies 1.5-3.0 mm. 8/sq.cm. tenacity 3
5.0% lactose	colonies pinpt. 8/sq. cm. tenacity 2	5.0% lactose (1.4×10^{-6} M amino acids)	colonies pinpt.-3.0 mm. 5/sq. cm. tenacity 2

Results from Second Set of Trials

Medium		Medium	
0.5% glucose	sheet of growth tenacity 1	0.5% glucose (7.5×10^{-3} M amino acids)	sheet of growth tenacity 3
1.0% glucose	sheet of growth tenacity 3	1.0% glucose (7.5×10^{-3} M amino acids)	thin sheet of growth tenacity 6
2.0% sucrose	sheet of growth tenacity 6	2.0% sucrose (7.5×10^{-3} M amino acids)	sheet of growth tenacity 4
5.0% sucrose	sheet of growth tenacity 5	5.0% sucrose (7.5×10^{-3} M amino acids)	thin sheet of growth tenacity 1
2.0% lactose	sheet of growth tenacity 6	2.0% lactose (7.5×10^{-3} M amino acids)	thin sheet of growth tenacity 7
5.0% lactose	no growth	5.0% lactose (7.5×10^{-3} M amino acids)	thin sheet of growth tenacity 5

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